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Diverse CTX Phages among Toxigenic *Vibrio cholerae* O1 and O139 Strains Isolated between 1994 and 2002 in an Area Where Cholera is Endemic in Bangladesh

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PCR surveillance of the *rstR* genes of CTX phages in *Vibrio cholerae* O1 and O139 showed no relationship between the incidence of disease and changes in the *rstR* but showed variations in their presence in O1 and O139 strains and the occurrence of multiple types in a few strains.

Of the 209 currently recognized serogroups of *Vibrio cholerae*, only strains belonging to serogroups O1 and O139 can cause cholera. Two major virulence gene clusters are now known to carry key virulence genes that are essential for the pathogenicity of *V. cholerae* O1 and O139. These gene clusters include the CTXϕ prophage (14), which carries the *ctxA* and *ctxB* genes (the genes that encode cholera toxin [CT], which is responsible for severe diarrhea), and the toxin-coregulated pilus (TCP) pathogenicity island, which carries genes for the biosynthesis of the TCP, required for colonization of the small intestinal epithelium (7).

The approximately 7-kb CTXϕ genome consists of the core and the RS2 region. The core region encodes proteins needed for the assembly and secretion of viral particles (Psh, Cep, $pIII^{CTX}$, Ace, and Zot) and also encodes CT, which is not necessary for phage morphogenesis (3), while the RS2 region represents a site-specific recombination system that allows lysogenic phage to integrate at a specific site on the host chromosome (14). The RS2 region of CTX prophage encodes proteins required for replication (RstA), phage integration (RstB), and regulation (RstR) of the lysogeny of CTXϕ (14). An antirepressor, *rstC*, is carried by a satellite phage, RS1, often present adjacent to the CTX prophage in toxigenic *V. cholerae* O1 El Tor and O139 strains (1, 5).

Diversity of the CTX phage repressor *rstR* has been described previously, and this diversity constitutes heteroimmunity among diverse CTX phages (8, 2). The difference in the *rstR* gene is also the only known genetic difference between any two different CTX phage types. The existence of at least four different *rstR* genes carried by different CTX phages, namely, CTX^{ET}, CTX^{class}, CTX^{Calc}, and CTX^{Env}, has been recognized (8, 2, 10). The epidemiological significance of the diversity of CTX phages is not clearly known, but at least two periods of explosive resurgence of cholera have been associated with strains showing changes in the *rstR* type of CTX phages. The first was the resurgence of *V. cholerae* O139 in August 1996 in Calcutta, India, which continued for a year (8, 9, 13), and the second was the resurgence of strain O139 in March to April of 2002 in Dhaka, Bangladesh (6). On the basis of their *rstR* genes and other phenotypic traits, genetic hybrids of classical and El Tor biotypes that cause cholera have been shown to exist, and these hybrids have been designated the Matlab variants of *V. cholerae* (11). To further document the distribution and temporal changes in the CTX phage contents of epidemic strains, we conducted a surveillance of CTX phage types by analyzing the types of *rstR* genes carried by a large collection of toxigenic *V. cholerae* strains.

We selected every 10th consecutive strain of *V. cholerae* O1 or O139 isolated from cholera patients admitted to the Matlab hospital, 50 km south of Dhaka, Bangladesh, from 1994 to 2002. A total of 169 strains of *V. cholerae* O1 and 95 strains of *V. cholerae* O139 isolated between 1994 and 2002 (with the exception of the year 1999) were included in this study. The procedure for the selective isolation of *V. cholerae* from stool samples of patients with acute secretory diarrhea and subsequent identification has been described in detail previously (12).

The serogroup of the strains selected were confirmed by using polyclonal O1 and O139 antisera. PCR was performed

TABLE 1. Oligonucleotide primer sequences used in PCR assays[a]

Gene	Primer sequence (5′–3′)	Amplicon size (bp)
ctxA (forward)	5′-CTCAGACGGGATTTGTTAGGCACG-3′	
ctxA (reverse)	5′-TCTATCTCTGTAGCCCCTATTACG-3′	308
rstR1 (forward)	5′-CTTCTCATCAGCAAAGCCTCCATC-3′	500
rstR2 (forward)	5′-GCACCATGATTTAAGATGCTC-3′	500
rstR3 (forward)	5′-CTGTAAATCTCTTCAATCCTAGG-3′	~300
rstR4 (forward)	5′-GTTAACGCTTCAAGCCTG-3′	400
rstA3 (reverse)	5′-TCGAGTTGTAATTCATCAAGAGTG-3′	

[a] Primers were for the detection of *rstR* and *ctxA* genes in *V. cholerae* O1 and O139 strains isolated from hospitalized patients in Matlab, Bangladesh.

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TABLE 2. Occurrence of the various *rstR* genes examined in this study among *V. cholerae* O1 and O139 strains

rstR gene(s)	Original nomenclature (reference)	No. of positive isolates	
		V. cholerae O1	*V. cholerae* O139
$rstR_1$	$rstR^{class}$ (8)	9	0
$rstR_2$	$rstR^{ET}$ (8)	141[a]	83
$rstR_3$	$rstR^{Calc}$ (8)	0	0
$rstR_4$	$rstR^{Env}$ (10)	0	0
$rstR_1$ + $rstR_2$	Not reported	6	0
$rstR_2$ + $rstR_4$	Not reported	6	6
$rstR_2$ + $rstR_3$	Combination (8)	0	3
$rstR_1$ + $rstR_2$ + $rstR_3$	Not reported	0	1
None[b]		7	2
Total		169	95

[a] Two strains were negative for the *ctxA* gene.
[b] None, negative for all of the *rstR* genes tested.

according to a previously described procedure (11). The primer sequences are shown in Table 1. *V. cholerae* O1 isolates (classical 154) and *V. cholerae* O139 (AR-196318) and *V. cholerae* non-O1 non-O139 (environmental SCE-188) isolates (10) were used as standard reference strains. We also used an *rstC* probe as described previously (4) to examine whether CTX prophage-negative strains, which show an *rstR* amplicon, carried RS1. The PCR products from five representative isolates (MJ1347, MM1079, MM2071, MP1950, and MP2044) were purified with a Microcon centrifugal filter device (Millipore Corporation, Bedford, Mass.), and a cycle sequencing reaction was performed with the same primers. DNA sequencing was performed by using standard conditions in an ABI PRISM 310 automated sequencer (Perkin-Elmer–Applied Biosystems, Foster City, Calif.). DNA sequence editing and analysis were performed with DNASTAR package 5.06 software.

Table 2 shows the distribution of different types of *rstR* genes among 169 strains of *V. cholerae* O1 and 95 O139 strains, isolated between 1994 and 2002 from hospitalized patients in Matlab, Bangladesh. We propose to designate the *rstR* genes with subscript numbers ($rstR_1$, $rstR_2$, etc.) since we anticipate that the number of such *rstR* genes that will be discovered in the future is likely to increase and thus a number designation is more suitable. The nucleotide sequences of 10 *rstR* amplicons from five isolates of *V. cholerae* O1 and O139 were similar to those of canonical *rstR* genes, with minor differences, as shown in Table 3.

Of the 169 O1 strains and 95 O139 strains, 9 and 2 strains, respectively, did not carry the *ctxA* gene and were considered nontoxigenic. Two of the nontoxigenic strains of *V. cholerae* O1, however, carried the $rstR_2$ genes. We further examined all nine *V. cholerae* O1 and two *V. cholerae* O139 strains with a probe specific for *rstC* to search for the presence of the RS1 element, which would explain the presence of the $rstR_2$ gene in the nontoxigenic *V. cholerae* O1 strains. However, only one of the two nontoxigenic *V. cholerae* O1 strains hybridized with the *rstC* probe; the other strain did not hybridize with the probe.

Three isolates from the year 1997 are of special interest. One of the isolates, MM004, is like the CTX-negative isolates of previous years, in that it was negative for an *rstR* gene of any type, *ctxA*, and *rstC*. The isolate MM1079, however, was positive for $rstR_2$ but negative for the *ctxA* gene and positive for *rstC*. Yet another 1997 isolate, MM2644, was $rstR_2$ positive but negative for both the *ctxA* and *rstC* genes. These strains might have undergone deletion in part of the CTX prophage. Overall, PCR results for the incidence of different *rstR* types in O1 and O139 strains showed no relationship between the temporal incidence of the disease and changes in CTX. However, the data presented do indicate variation in the incidence of *rstR* types, their presence in O1 and O139 strains, and the infrequent but interesting occurrence of multiple types in some strains. The *rstR* gene offers a window to assess the evolution of the phage.

Nucleotide sequence accession numbers. The nucleotide sequence accession numbers of 10 *rstR* amplicons from five isolates of *V. cholerae* O1 and O139 have been submitted to GenBank under accession numbers AY704650 to AY704659.

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TABLE 3. Nucleotide sequences of various *rstR* genes of *V. cholerae* O1 and O139 isolates from Matlab, Bangladesh, in comparison with corresponding sequences in GenBank

Strain (gene)	Serogroup	GenBank accession no.	Mutation comparison (accession no.)
MJ1347 ($rstR_1$)	O1	AY704650	Identical to the *V. cholerae* 569B repressor *rstR* (AF055890)
MJ1347 ($rstR_2$)	O1	AY704651	Silent substitution of C to T at position 1938 compared to the *V. cholerae* O1 biovar El Tor N16961 transcriptional repressor *rstR* (AE004224)
MM1079 ($rstR_2$)	O1	AY704652	Silent substitution of C to T at position 1938 compared to the *V. cholerae* O1 biovar El Tor N16961 transcriptional repressor *rstR* (AE004224)
MM2071 ($rstR_2$)	O1	AY704653	Silent substitution of C to T at position 1938 compared to the *V. cholerae* O1 biovar El Tor N16961 transcriptional repressor *rstR* (AE004224)
MM2071 ($rstR_4$)	O1	AY704654	Silent substitution of A to G at position 375 and C to T at position 452 compared to the *Vibrio* phage CTX RSTR (*rstR*) (AY145127)
MP1950 ($rstR_1$)	O139	AY704655	Identical to the *V. cholerae* 569B repressor *rstR* (AF055890)
MP1950 ($rstR_2$)	O139	AY704656	Silent substitution of C to T at position 1938 compared to the *V. cholerae* O1 biovar El Tor N16961 transcriptional repressor *rstR* (AE004224)
MP1950 ($rstR_3$)	O139	AY704657	Identical to the *Vibrio* phage CTXϕ Calcutta *rstR* (AF133310)
MP2044 ($rstR_2$)	O139	AY704658	Silent substitution of C to T at position 1938 compared to the *V. cholerae* O1 biovar El Tor N16961 transcriptional repressor *rstR* (AE004224)
MP2044 ($rstR_3$)	O139	AY704659	Identical to the *Vibrio* phage CTXϕ Calcutta *rstR* (AF133310)

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